Declaration of Pieter Pouwels

I, Pieter Pouwels, on oath declare and sayeth that:

Introduction

1. I am the same Pieter Pouwels who is named as a co-inventor on US patent application No. 10/500,307. I make this Declaration in support of that Patent Application. My *curriculum vitae* is attached. As can be seen from my *curriculum vitae,* I have worked, and published, extensively in the field of expression of heterologous proteins in bacteria and that of vaccine development.

2. I have reviewed the above-identified application and understand the nature and scope of the invention claimed therein. I have also reviewed the Official Action issued by the US Patent and Trademark office (US PTO) on 15 June 2007 for this application. I understand that the claims stand rejected as lacking enablement, for inadequate written description and for indefiniteness. This Declaration is now submitted to outline how the specification provides ample information to allow the invention to be put into practice and also adequate written description demonstrating that we, the inventors, were in full possession of the invention. In addition, the Declaration outlines how the person in the field would have understood the terminology used in the claims and hence there is no indefiniteness.

Enablement

3. The specification provides adequate information to put the invention into practice. The specification provides a detailed description of how the modified S proteins from *Lactobacillus* bacteria can be generated and assessed for their ability to form a crystalline monolayer as specified by the claims. As discussed below, I believe that the non-enablement rejections may have arisen from misinterpretation of the claims and that the person in the field, reading the specification, would not interpret the claims in that way.

4. The Official Action comments on a number of parameters in relation to enablement from the Wands decision. I have set-out my comments on each of the factors outlined in the Official Action below.

(i) Nature of the invention

5. I think that a misunderstanding as to the meaning of the term "*crystallisation*" may have arisen contributing to the non-enablement rejection. In particular, reference in the claims to ability to crystallize, refers to the ability of the modified protein to form a two-dimensional monolayer, that, in a non-modified form, comprises the surface layer that envelopes the entire bacterial cell. It does not refer to the ability to form a three dimensional array, for example as in crystals of salt growth or of proteins other than S proteins from an aqueous solution, as the Official Action appears to be interpreting the expression to mean. That is not how the person in the field would interpret the claim.

6. Thus, reference to a crystalline structure in the present instance does not refer to the formation of the type of crystal that is used to determine the three-dimensional structure of a protein in X-ray crystallography and the difficulties of generating such three dimensional crystals that is commonly associated with X-ray crystallography.

7. S layer proteins naturally form two-dimensional crystalline monolayers (or arrays), that are present as an envelope on the surface of bacteria or in the form of a sheet in the absence of bacteria, and that is what the claims refer to. Indeed, formation of a two-dimensional monolayer can, in fact, make it impossible for the S-layer protein to form a three-dimensional crystal of the type used in X-ray crystallography. Given that the specification deals with S proteins, the ordinary reader would understand that is what is meant by reference to crystallisation in the claims and the specification gives ample illustration to determine whether or not a particular modified S protein has such properties, as further setout below. Indeed, the claims have been amended to make that explicit.

(ii) *Breadth of the claims*

8. I also feel that confusion may have arisen as to what the term "*S protein*" defines. The surface layer protein is defined by its ability to form a regular two-dimensional structure at the outside of a bacterium, composed of one sub-unit, the S-layer protein. An S-protein, thus, defines a protein present at the surface of bacteria that has the capacity to form a regular two-dimensional monolayer, either at the bacterial surface or *in vitro* (in the absence of bacteria) this is outlined in Sleytr, U and Messner, P *An Rev Microbiol* (1983) **37**: 311-339 now submitted.

9. The term S protein therefore defines a narrow subset of proteins with one S protein typically being found for each bacterium. The term does not refer to any and all proteins found in the bacterial cell membrane or bacterial cell wall. The present claims are not therefore directed to hugely broad array of proteins. They are directed to a very specific, tightly related, protein type, fulfilling the same role in each bacterium. That is particularly the case now the claims have been amended to refer to the protein coming from a *Lactobacillus*. In addition, the specification provides the necessary tools to assess the key property of any modified S protein from a *Lactobacillus* to form a regular two-dimensional monolayer that underpins the invention.

10. Given the tightly linked nature of the group of proteins claimed and the teaching of how to determine if a modified protein can form a crystalline monolayer, the breadth of the claims is reasonable and no difficulty would be encountered in putting the invention into practice across the breadth of the claims. That is particularly the case following limitation to the unmodified S protein coming from a *Lactobacillus*.

(iii) *Direction of guidance presented in the specification*

11. Contrary to what is stated in the Official Action, the specification is not silent with regard to which bacterial surface protein will crystallise, or with regard to how to practice the claimed invention. On the contrary, the specification describes the nature and properties of the modified S-proteins and how to determine whether or not a modified *Lactobacillus* S protein can form a crystalline monolayer as specified by the claims.

12. Pages 44 to 60 of the specification describe in detail how DNA sequences coding for S-layer proteins with internal insertions of heterologous amino acid sequences can be constructed and how such sequences may be expressed in different bacterial species. These passages of the Examples provide illustrative instances of insertions resulting in the formation of a modified S-protein that is still able to form a regular two-dimensional crystalline

monolayer. The functional analysis of the modified S-layer proteins described at pages 45 to 48 demonstrates the ability of the modified proteins actually generated to form monolayers sheets. Thus, the skilled person can generate and assess modified S proteins using the instructions provided.

13. Of particular relevance is the information on pages 45 and 46 of the specification, particularly Table 1 on page 45 of the specification. This shows that insertions at five different locations led to modified *Lactobacillus* S proteins that retained the capacity to crystallize (form a regular two-dimensional monolayer) as specified by the claims. The skilled person is therefore able to identify those insertions for any given heterologous polypeptide that will result in a modified *Lactobacillus S* protein retaining the ability to form a crystalline monolayer.

14. The specification therefore describes a simple assay; the formation of a precipitate upon dialysis of hybrid S-proteins expressed in *E. coli*, which can be used to identify or confirm modified *Lactobacillus* S layer proteins retain the ability to form S layers. The specification also describes how electron microscopic analysis of the protein precipitates can be used to confirm ability to crystallise. Assessing ability to crystallise is therefore a routine procedure given the information provided by the specification and should not be confused with the much more difficult process of forming three-dimensional crystals for X-ray crystallography.

15. The specification therefore provides a detailed description of how a person can assess the "crystallization capacity" involving simple routine techniques, which are well within the capabilities for the ordinary person in the field.

16. Thus, in the specification we describe simple experimental methods to determine the capacity of the Surface layer protein to form a regular two-dimensional crystalline monolayer and thus to be functional as an S-layer. This allows easy evaluation of any modified surface layer protein with any insertion of a heterologous peptide for its capacity to form a two-dimensional crystalline monolayer. The specification does therefore provide adequate guidance to allow the invention to be put into practice.

(iv) *Presence or absence of working Examples*

17. The Examples presented in the application show five different modified *Lactobacillus* surface layer proteins demonstrating the capacity to form regular two-dimensional crystalline monolayers. Working examples are therefore provided and the invention is reduced to practice.

18. The Examples illustrate the invention with particular modified *Lactobacillus* S proteins with internal insertions where the S-layer protein is still capable of forming a two-dimensional crystalline monolayer as specified by the claims. Simple, standard procedures to construct such hybrid proteins from the unmodified S proteins of *Lactobacillus* bacteria are described. The Examples show the reader how the invention can be put into effect using actual working examples.

(v) *State of the prior art*

19. Nothing in Bowie *et al* prevents the invention being put into practice. The key to the present invention is the finding that modified S proteins with heterologous proteins inserted can still retain their ability to form crystalline two-dimensional monolayers.

20. Bowie *et al* is concerned with whether insertion of foreign amino acid sequences in a protein will affect the protein's functional properties by changing the three-dimensional structure of the protein and change its ability to form a three-dimensional crystal of the type used in X ray crystallography. That is not of relevance of the ability of the modified S protein to form a two-dimensional crystalline monolayer as specified by the claims. Bowie *et al* does not therefore cast any doubt on the ability of the invention to be put into practice.

(vi) *Quantity of experimentation necessary*

21. As set forth above, the Example provides a simple means to determine if a modified *Lactobacillus* surface layer protein falls within the scope of the invention, i.e. the ability to form a two-dimensional crystalline monolayer. The simple test taught by the specification and Examples does not represent undue experimentation and the skilled person is readily able to both generate modified S proteins from the unmodified proteins of *Lactobacillus* bacteria and to assess their ability to form two-dimensional crystalline monolayers, as specified by the claims.

(vi) *Summary*

22. The various criteria laid down are satisfied. The specification teaches how to put the invention into practice, including providing actual examples of modified *Lactobacillus* S proteins as claimed.

Written Description

23. As I understand it, the claims are now limited to modified S proteins where the original S protein was from a *Lactobacillus* bacterium. As discussed above, the specification describes five actual modified *Lactobacillus* bacterial S proteins which retain the ability to crystallise to form a two-dimensional monolayer after the insertion of a heterologous polypeptide. An adequate number of representative examples of the modified proteins claimed are therefore described in the specification itself.

24. S proteins from *Lactobacillus* bacteria represent a tightly defined group of proteins. The specific modified S proteins described in the specification do therefore provide a representative and adequate illustration of the invention. Furthermore, the specification describes the necessary tests to show that a given modified *Lactobacillus* S protein can form a crystalline monolayer as specified by the claims as also discussed above.

25. The specification does describe the S proteins in detail. Pages 1 and 2 of the specification define the surface layer protein as a particular protein that is able to form a regular structure. Pages 11 to 16 describe the *Lactobacillus* bacterium that possess such an S-layer, including *Lactobacillus acidophilus*.

26. Possible insertion sites are described on pages 6 and 7, whilst examples of heterologous polypeptides that can be inserted are given on pages 24 to 26 of the application. These should provide adequate written description. Moreover, the Example shows insertion of a heterologous peptide in five different locations in a *Lactobacillus* bacterial S protein that retain the ability to form a two-dimensional crystalline structure and hence fall within the defined group as indicated by the claims. These represent the "*representative number of S-proteins with inserted heterologous proteins that can still crystallize*" referred to in he Official Action.

27. It is reasonable to extrapolate from the results seen in the Examples to S proteins from *Lactobacillus* bacteria in general given that such S-proteins have generic properties. That is apparent from various literature reviews such as Sleytr, and Messner, (1983) *An Rev Microbiol,* **37** 311-339; Boot and Pouwels, (1996) *Mol Microbiol.* **28** 1209-1213. S- proteins share a number of common characteristics which are not found in non S-layer proteins, the most prominent one being the capacity to form a regular two-dimensional array on the surface of bacteria or *in vitro* (in the absence of bacteria). These special features allows the extrapolations from the S-protein of *L. acidophilus* dealt with in the Examples to other S-proteins from *Lactobacillus* bacteria to be made.

28. The overall structure of S-proteins is essentially the same. They are composed of two domains, one responsible for attachment of the S-protein to the bacterial cell wall (in the case of *L. acidophilus* this is the SAC domain described on page 3, bottom lines), and a second domain that is responsible for crystallization (called SAN in the S-protein from *L acidophilus,* see page 4). Consequently, it would be evident to the skilled person that what holds for the S-protein from *L. acidophilus* described in the Examples, also holds for all other S-proteins from *Lactobacillus* bacteria.

29. Primary and secondary sequence analysis reveals that the S-proteins from these bacteria are similar to the extent that they are expected to have the same folding.

30. The fact that other S-layer proteins behave in a similar manner is also strengthened by the observation of the literature publication Jaaskelainen and Palva (*Appl. Environ. Microbiol.* 68 (12), 2002, pp 5943-51), published after the filing date of the application. Jaaskelainen and Palva demonstrates insertion of "foreign epitopes" (heterologous polypeptides) in the surface layer results in surface display of these epitopes and leaves the S-layer as such intact.

31. The specification does therefore provide adequate and representative examples of modified *Lactobacillus* bacterial S proteins retaining the ability to crystallise. The specification also describes methods for the generation of such modified proteins as well as ways to confirm that a given polypeptide can crystallise.

Definiteness and terminology used in the field

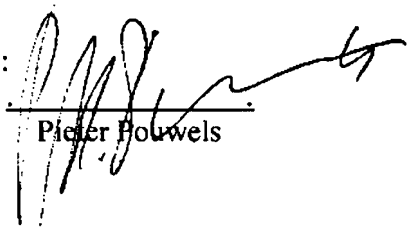
32. I understand that the term "pI" in claim 6 has been rejected as being vague and indefinite. However, the term "pI" is one frequently used in the art and refers to the isoelectric point of a protein, i.e. the pH at which a protein has no net charge. Those working in the field would therefore understand what is meant by the term.

33. In respect of the rejection to the expression "*an antigen causing or specific for a disease*", the terminology would again be clear to the person in the field. In particular an

antigen causing a disease is one responsible for the disease itself, such as an auto-antigen responsible for the development of an autoimmune disease. In contrast, an antigen specific for a disease is one, for instance, representative of a pathogen responsible for a disease. Those working in the field would therefore be able to understand what is meant by the claims.

34. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. Further, that these statements are made with knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application or any patent issuing thereon.

Dated 12/11/2007

Signed: 

Pieter Bouwels

CURRICULUM VITAE

Name	Peter H. Pouwels
Date of birth	7 september 1935
Nationality	Dutch
Education	Biochemistry and molecular biology; Dissertation in 1965 (Leiden University)
Positions	Head Department Molecular Genetics and Gene Technology TNO (1971-1995) Professor of Molecular Gene Technology at University of Amsterdam (1990-2000) Consultant for Lactrys (since 2000)

Research

1961 - 1969	the replicative form of the *E. coli* bacteriophage OX174.
1965 - 1966	postdoctoral year with Paul Berg (Stanford, USA) on the mechanism of missense suppression in *E. coli*.
1969 - 1980	control of transcription of the tryptophan operon of *E. coli*.
1980 - 1994	development of gene-transfer and gene-expression systems for filamentous fungi, bifidobacteria, yeasts, animal cells, insect cells and *E. coli*.
1980 - 1988	FMDV and polio subunit vaccine development.
1985 - 2000	Lactobacillus research concentrated on i) the role of S-layer proteins and other cell-associated proteins in adhesion, ii) the potential of lactobacilli as mucosal delivery vehicles for oral vaccination, iii) carbon fluxes and carbon catabolite repression in lactobacilli

Publications More than 120 research articles; review articles; chapters in books; etc